Life history of shanny *Lipophrys pholis* in the Portuguese occidental coast: new insights from otolith microstructure

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Abstract

Lipophrys pholis is a Blenniidae interdital fish usually found in the Portuguese coastal shores. Information available on the ecology of this species is scarce and is mainly from reproductive and behavioral studies, made in some cases from captivity experiments. Spawning takes place from October/November to May in rocky nests, after which demersal eggs hatch and larvae disperse to the coastal area. Early juveniles (recruits) return within two to three months, apparently to a particular set of rock tide pools, suggesting a homing behavior. In the present study, and to assess the primary increment periodicity in otoliths of early juveniles of *L. pholis,* 90 individuals, ranging from 16 to 39 mm of total length, were captured in a rocky beach in north of Portugal (Cabo do Mundo) and reared in laboratory. Otolith direct validation procedures using fluorescent dyes (alizarin and tetracycline) showed a daily deposition of the primary increments, suggesting that they are reliable sources of age information for *L. pholis*. Additionally 91 recruits (TL≤30 mm) were collected using a hand net in April/May 2013 during the low tides periods in three rocky beaches along the occidental Portuguese coast (Cabo do Mundo, Peniche and Vale do Homem). The purpose was to estimate the total and recruitment ages by counting the primary increments and by tracking the settlement mark, respectively. In the lab, total length of individuals has recorded and sagittal otoliths were extracted. The relationship between fish length and otolith size (radius and diameter) was explored. After adequate preparation, otoliths were observed in light microscopy. Age ate recruitment ranged between 57 and 73 days, showing a latitudinal pattern of temporal recruitment probably related with the seawater temperature. Future works using microstructural and microchemistry analysis from otoliths of individuals collected in different life stages (e.g. embryos, recruits and adults) can be useful to assess connectivity between the spawning and recruitment areas in *L. pholis.*

Key words: blennies, life history, connectivity, sagittae, daily rings

Resumo

Lipophrys pholis é um peixe interdital normalmente encontrado nas zonas costeiras portuguesas e que pertence à família Blenniidae. A informação disponível sobre a ecologia desta espécie é escassa, e resulta principalmente de estudos de reprodução e comportamentais, realizados, em alguns casos, a partir de experiências em cativeiro. A postura ocorre de Outubro/Novembro a Maio em ninhos construídos em praias rochosas, mas depois da eclosão dos ovos demersais, as larvas são dispersas através das correntes pela zona costeira. Os primeiros juvenis (recrutas) regressam dentro de dois a três meses, aparentemente para um conjunto particular de poças rochosas, o que sugere para a espécie um comportamento típico do "homing". Neste estudo 90 recrutas, entre 16 e 39 mm de comprimento total, foram capturados numa praia rochosa do norte de Portugal (Cabo do Mundo). Para avaliar a periodicidade de deposição dos incrementos primários nos otólitos recorreu-se a marcadores fluorescentes (alizarina e tetraciclina) que demostraram uma deposição diária dos incrementos primários nos otólitos, sugerindo que os otólitos podem ser utilizados com sucesso para estimar a idade de *L. pholis*. Adicionalmente, foram capturados, com recurso a um camaroeiro, 91 recrutas (TL ≤ 30 mm), em Abril / Maio de 2013, durante as marés baixas em três praias rochosas ao longo da costa ocidental Portuguesa (Cabo do Mundo, Peniche e Vale do Homem). Estimou-se a idade total e a idade de recrutamento contando os incrementos primários e tendo em linha de conta o posicionamento da marca de assentamento, respetivamente. No laboratório, foi medido o comprimento total dos indivíduos e foram extraídos os otólitos. A relação entre o tamanho dos indivíduos e dos otólitos (raio e diâmetro) foi analisada. Após preparação, os *sagittae* foram observados com um microscópio de luz. A idade no recrutamento costeiro variou entre 57 e 73 dias, sugerindo um padrão latitudinal de recrutamento temporal, provavelmente relacionado com a temperatura da água do mar. A análise micro-estrutural e microquímica futura de otólitos de indivíduos capturados em fases de vida diferente (por exemplo, embriões, recrutas e adultos) poderá ser utilizada com sucesso no futuro para avaliar a conectividade entre as zonas de postura e as áreas de recrutamento em de *L. pholis*.

Palavras-chave: blenídeos, ciclo de vida, conetividade, sagittae, anéis diários

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Abbreviations

ICP-MS: Inductively Coupled Plasma Mass Spectrometry

LM: Light Microscopy

SEM: Scanning Electron Microscopy

SM: Settlement Mark

TL: Total Length

1. Introduction

1.1. Present knowledge about the *ecology of Lipophrys pholis*

The shanny*, Lipophrys pholis* (Linnaeus, 1758), is a common intertidal fish found in the Portuguese coastal shores that belongs to the Blenniidae family. It is one of the most abundant fishes in the NE Atlantic, from Mauritania to Norway, including the Azores and Madeira Islands, but also in the Mediterranean (Zander, 1986; Almada *et al*., 2001) (Fig. 1). *L. pholis* has been studied intensively in the northern part of its geographical range, especially around the British Isles (Qasim, 1957; Bowers *et al*., 1960; Dunne, 1977; Shackley & King, 1977; Milton, 1983). Blenniids are most abundant in the tropics, and most European species breed during spring and early summer (Zander, 1986). The majority of them either extends their ranges to West Africa or are specially abundant in the Mediterranean Sea. The geographic distribution of *L. pholis* comparatively to other European blennids reaches much higher latitude because it is probably a species adapted to cooler waters (Almada *et al*., 1990a).

In temperate waters, blennies are among the best-adapted species to turbulent waters subject to wave action (Gibson, 1986; Horn & Gibson, 1988). At higher latitudes, the reproductive season tends to start later and to end sooner, when conditions become favorable for larval dispersion and juvenile growth. The end of the breeding season should be triggered by a short temporal window that allows late-born juveniles to reach a size that ensures high probabilities of survival during winter. *L. pholis* occurring at low latitudes have a protracted breeding and recruitment seasons (Conover, 1992). This lengthening is mainly due to a marked advance in the onset of breeding (Faria *et al*., 1996). In Great Britain, *L. pholis* breeds during spring and early summer (Qasim, 1957) while in Portugal the breeding season occurs in the cooler months, from October/November to May (Almada *et al*., 1990a; Faria *et al*., 1996).

Figure 1 - Distribution map of *L. pholis* [modified from Zander (1986) and Almada et al., (2001)].

L. pholis is phylogenetically closer to *L.* (= *Paralipophrys*) *trigloides* and to *Coryphoblennius galerita* than to the other small *Lipophrys* species, which form their own independent monophyletic group (Bock & Zander, 1986; Almada *et al*., 2005). A close relationship between *L. pholis*, *L. trigloides* and *C. galerita* has already been proposed by other authors based on osteological and karyological data (Papaconstantinou, 1977; Bock & Zander, 1986; Garcia *et al*., 1987). Data from molecular studies found that *L. trigloides* and *L. pholis* are sister species, while other small *Lipophrys* species formed a very distinct clade, questioning the monophyly of the genus *Lipophrys* which, according to the authors, must include only *L. pholis* and *L. trigloides* (Almada *et al*., 2005).

L. pholis has morphology characteristics typical of other blennies, such as a smooth and elongated body, with a large and blunt head, fairly large eyes and small tentacles bellow the eyes, but without head tentacles and its eyes are set high up on its head (Zander, 1986; Arruda, 1979) (Fig. 2). *L.pholis* eggs are golden-brown,

transparent with a spherical shape and the newly hatched larvae present fully pigmented eyes, with the anus and mouth already open. At hatching larvae present peritoneal pigmentation and twelve rows of melanophores on the pectoral fins. Ventrally there were 2-4 melanophores on the throat and 7-9 on the last myomeres. Dorsally, there were some sparse melanophores over the brain and upper lip and there was one melanophore between the inner ear vesicles. After metamorphosis the juvenile fish developed pigmentation. A ventral row of melanophores at the base of the anal fin was present and the other fins were also pigmented. The head was extremely pigmented and there was some pigmentation at the throat. Dorsally there were dark bands that extended through the midline, and alternated with three other blotches situated laterally (on each side of the body). The pigmentation pattern is maintained during development with an increase in the number and intensity of melanophores at the ventral row, and at the cephalic region, with melanophores extending from between the eyes to the dorsal region. During spawning, the males present a general black coloration with white lips, while the females show a light coloration, with fins almost transparent (Faria *et al*., 2002).

Figure 2 - Photography of an adult *L. pholis.(personal captation)*

L. pholis can live in a variety of substratum types during its life cycle. Juveniles occur in shallow pools of simpler topography, but when they grow they tend to leave the pools and begin to seek larger crevices, spaces under stones and other protected microhabitats (Qasim, 1957; Faria & Almada, 2001b). Juveniles and adults clearly prefer rocky to sandy substrata. It is likely that this preference could represent a better protection from both predators and waves action. The relative abundance of the individual size groups differ according to the tidal levels (Qasim, 1957). The study of

the pattern of spatial distribution and behavior of an intertidal fish assemblage on a rocky intertidal platform in the south of Portugal during high tides, showed that *L. pholis* is specifically abundant in the upper intertidal and were subject to larger displacement, up and down, with the tide (Faria & Almada, 2006). However it is suggested that the species could move into deeper waters during the winter season (Gibson, 1967).

The intertidal fishes show patterns of courtship and agonistic behavior that minimize the loss of contact with the substrate, a feature that is probably adaptive in conditions of marked turbulence (Almada & Santos, 1995).

Juveniles of *L. pholis* could defend the access to shelter holes existent within their home range habitat, but without exclusive use (Gibson, 1968). Usually *L. pholis* use a network of familiar pathways that included more than one shelter (Almada *et al.,* 1983). The individuals would compete not for a permanent presence in a shelter, but for undisputed priority of access to the shelters present in the neighboring areas, to minimize the time during which the fish is exposed to potential predators. In the case of the breeding males, the “diffuse territoriality” would change to a more traditional form of territorial defense, because they tend to concentrate their visits on a single hole and restrict their movements to the surroundings areas. The findings that fishes removed from pools and released several meters away were found in their original pools support the hypothesis that when in pools, fishes acquire information on the exact locations of shelters (Qasim, 1956a; Almada *et al*., 1983, 1990b; Almada & Santos, 1995).

In the marine intertidal zone, fishes from the families Gobiidae and Blenniidae are believed to have mapping orientation abilities (Arondson, 1951; Gibson, 1968; Almada *et al*., 1983). Some rocky intertidal fish have been shown to have good homing abilities (Gibson, 1967; Santos *et al*., 1989; Mitamura *et al.,* 2005). Homing behavior has been generally defined as the ability of an animal to return to a spatially restricted location that it previously occupied following displacement to an unfamiliar site (Williams, 1957; Gerking, 1959; Papi, 1992). One of the key benefits of homing is that it ensures an animal returns to a familiar location (White & Brown, 2013).

Homing is likely to be particularly important in extreme environments, where conditions fluctuate greatly over space and time. In intertidal fish, for example, survival may be influenced by an individual’s ability to return to its home rock pool after feeding excursions took place during the high tide periods and thereby avoid being stranded in unsuitable areas at low tides (Williams, 1957).

Adults of *L. pholis* were able to orient themselves toward their home areas even if they were placed in an unfamiliar distant area, by relying on cues available in the unfamiliar area (Jorge *et al.,* 2012). Individuals of *L. pholis* are able to easily find a

refuge in a novel habitat by quickly swimming toward the nearest dark area. They are also able to memorize the position of a refuge based on conspicuous visual cues (Dodd *et al.,* 2000). *L. pholis* are able to memorize and use spatial maps of local landmarks to learn the position of a refuge (Burt de Perera & Guilford, 2008). Males are territorial and defend their nests against conspecific competitors or potential predators, by staying within the same pool or set of pools for extended periods of time (Gibson, 1968; Faria & Almada, 2006). *L. pholis* nests are often placed in intertidal holes or crevices that become completely immersed for extended periods of time during low tides (Faria & Almada, 2006).

In situ observations show that the daily average feeding time of *L. pholis* is relatively short (5h34min) (Almada *et al*., 1992) which corresponds to the daylight time period when territories are underwater (Gonçalves *et al*., 1998). During this period, individuals swim actively in search of food and then return to the pools where they stay during low tide (Arruda, 1979). Therefore, the similarity between the diet of blenniids caught in different pools is expected. When juveniles of *L. pholis* reach a total length (TL) of 70 mm they initiate a radical shift in their pattern of microhabitat occupation (Faria & Almada, 2001a). During the ontogenetic development of *L. pholis*, a quantitative and qualitative alteration of the feeding regime was clearly visible. Some prey items, important for the smallest size classes, tended to disappear from the diets of larger individuals, being progressively replaced by different prey items (Monteiro *et al*., 2005). All the prey, caught by *L. pholis,* are invertebrates. Mollusca are the most highly represented taxon, namely gastropods, followed by bivalves and, to a lesser degree, polyplacophorans. Cirripede crustaceans are highly abundant, however only in some occasions appear in their diet. Other well represented crustaceans are isopods and amphipods (Monteiro *et al*., 2005).

In winter, diet species richness is poor, with only 24 prey taxa recorded. Diet species richness (37 taxa) increases considerably in spring and also during the summer and autumn months. In general, *L. pholis* does not specialize in any of the mentioned groups, although some of them tend to show higher frequencies in the diet. Temporal analysis of the diet does not show any significant differences throughout the year. However there is a greater species richness and diversity found in the feeding pattern of the specimens captured in spring and summer, a moment coinciding with the settlement of many species of invertebrates which, moreover, have the appropriate size to be ingested by the blenniids (Mazé *et al*., 1999).

The rocky intertidal habitat, however, is a hostile environment with its regular alteration of physical conditions, mainly due to more or less prolonged periods of

emersion and submersion (Lewis, 1964; Barlow, 1999), forcing specific adaptations in its colonizing fauna and flora. Nevertheless, many independent fish phyletic lines have settled in this particular habitat with their reproductive behavior and life history falling into a narrow range of strategies (Gibson, 1982, 1999; Almada & Santos, 1995; Zander *et al.,* 1999).

During the breeding period the males establish territories in crevices (or space between stones) where spawning takes place (Qasim, 1957; Dunne, 1977; Almada *et al*., 1990b). Studies made in captivity, shows that spawning lasts more than 9 hours (Faria *et al*., 2002). The male first the rubs the substratum with the genital papilla and the female follows the male´s path while laying eggs, suggesting that the female spawns over the surface that is likely to already contain sperm. The nests contain more than one batch of eggs from single or different females, so the stage of development varied between the different batches, showing that they had been deposited at different times (Qasim, 1956a). During this stage males exhibit a typical dark coloration pattern, and defend and ventilate the developing eggs until hatching (Qasim, 1956a; Almada *et al*., 1990b, 1992, Faria *et al.,* 2002).

The active defense of the area around the nest probably serves, at least, two functions: keeps potential egg predators and other intruders away from the nest; and minimizes feeding activities of conspecifics in the area surrounding it (Almada *et al*., 1992). The unguarded egg masses of *L. pholis* may survive for up to 5 days without predation (Almada *et al*., 1992).

A rearing experiment showed that the embryonic developmental sequence starts with the differentiation of the embryo at day 2 after fertilization, the presence of eye rudiments at day 4, and the formation of myomeres and heart beatings at day 5. Embryonic development lasts 16 days at 17ºC. Larvae began to settle 29 days after hatching (13-14 mm) and 8 to 9 days later they were benthic (15-16 mm). However, juvenile behavior such as turning movements of the head and hiding under objects in close contact with the surfaces by flexing the body against them was observed only at 17-19 mm individuals (Faria *et al*., 2002).

After hatching, the larvae swim actively and the onset of exogenous feeding occurs one day later. The larvae of *L. pholis* have a long planktonic life (one to two months) (Qasim, 1955). After metamorphosis and settlement the juveniles show typical behaviors associated with a benthic mode of life, such as lateral movements of the head and hiding behaviors, which could be important for survival in a highly irregular substrate (Faria *et al*., 2002).

Juveniles recruited in early winter in Portugal, are probably able to reach the minimum size necessary to reproduce in the next season, since they could grow continuously for several months (Faria *et al*., 1996). Recruitment of fish < 20 mm ceases 3 months after the end of the breeding season. Recruitment is an operational process: that refers to the first age class in a population (0^+) which includes the settlement and survival of settled individuals, and is influenced by biological events occurring during the planktonic stage and settlement processes, as well as post-settlement mortality (Keough & Downes, 1982 Faria *et al*., 1996).

Larval availability and post-recruitment processes in rocky intertidal invertebrate populations have a relative importance. It means that in a few cases larval supply may limit population size, but in others, larval supply is more than sufficient to ensure population renewal (Connell, 1985). The predominant mechanism that limits population size is density dependent post-recruitment mortality, which also seems to occur with *L. pholis* (Connell, 1985).

The photoperiod and temperature can affect the synchronism of the annual cycle of gonadal maturation in *L. pholis* (Shackley & King, 1977). The temperature appears to be an important abiotic trigger stimulus for the onset of reproduction in this species (Almada *et al*., 1990a). For species with an extended reproductive season, such as *L. pholis*, it is probably important to limit the duration of the each breeding season in order to restore the nutritional reserves and to allow growth of the adult itself, since fecundity tends to increase with the individual body size (Wootton, 1990).

At higher latitudes the embryos remain in a dormancy stage throughout the coldest months, being released in the spring when conditions are favorable for feeding and growth of the larvae (Crisp, 1954). This behavior suggests a principle that may be generally applicable to all marine animals which have an oceanic planktonic larval stage. The breeding cycles are highly regulated in a way that the larvae hatch during the season in which is most favorable for finding planktonic food (Faria *et al.,* 2005).

The previously papers provide important information about the shanny concerning its reproductive biology, ecology and behavior. However the knowledge about the population structure, fish movement patterns and connectivity is, at present, scarce. *L. pholis* could be used as a model in biology to understand connectivity of marine populations, monitor dispersal in planktonic fish larvae and to study fish coastal recruitment processes.

1.2. Otolith microstructure and microchemistry as an useful ichthyological tool

Important information for ecological research and fisheries management is obtained from fish otoliths. Otoliths are inner ear stones, made of calcium carbonate ($CaCO_3$) that play an important role in balance and auditory reception of teleost fish (Popper & Platt, 1993). The $CaCO_3$ is crystallized mainly in the mineral form of aragonite (Carlström, 1963) due to the action of an organic matrix (otolin) in which acidic amino acids predominate (Degens *et al*., 1969).

There are three pairs of otoliths (sagittae, lapilli and asterisci) encapsulated within the otic vesicles of the fish head which vary considerably in size, also being formed in different ontogenetic stages. The sagittae and lapilli generally form earlier in development than the asterisci, which in some species do not form until after hatching. The sagittae are most often used otoliths for ageing purposes and other analyses because they are the largest, earliest formed and easiest to extract of the three pairs (Green *et al*., 2009).

Otolith formation starts with a primordium, which is generally the first calcified tissue in the embryo (Dunkelberger *et al*., 1980). The nucleus is formed when the first discontinuous unit (Dunkelberger *et al.,* 1980) is laid down which corresponds to hatching, first feeding, or start of activity (Brothers & McFarland, 1981; Morales-Nin, 1992), although some species with long embryonic periods may start forming increments before hatching (Morales-Nin, 2000).

Otolith microstructure can provide useful information on life history traits of fish allowing to determine pelagic larval durations, to reconstruct settlement patterns and to investigate information provided by annual and daily increments (Choat & Robertson, 2002; Thorrold & Hare, 2002). Age determination of fishes based on periodic growth increments in otoliths has become a routine tool in fisheries science over the last century. Over 1 million fish were likely estimated through otoliths by fisheries scientists around the world (Campana & Thorrold, 2001). The structure of otoliths is particularly useful to reveal ontogenetic or environmental patterns changes experienced by individual fish. Specific variation in the environment can induce a specific mark in the otolith structure which could be useful afterwards as a reference mark to compare individual life history trait responses (Sponaugle & Pinkard, 2004).

The biological significance of first mark in otoliths is probably species specific and may correspond to several life history events such as yolk-sac absorption, hatching or first feeding (Wright *et al*. 2002b). In some species, such as tropical clupeids (e.g. *Stolothrissa tanganicae and Limnothrissa miodon),* otolith growth may

initiate increment deposition at yolk sac absorption up to 2–8 days after hatching (Kimura, 1995). The next major steps for a developing fish can be metamorphosis (i.e., the transition between larval and juvenile life) and then settlement (i.e., final installation in one specific environment), a particularly sensitive stage in tropical areas and especially for reef species (Leis, 1991).

The metamorphosis stage varies greatly between species but it is generally a strong transition associated with the end of ossification and for that reason it constitutes a tremendous physiological and morphological stress for the fish. Metamorphosis also can be associated with habitat change including changes in environmental conditions and behavioral changes, for example in mesopelagic marine species (Myctophidae, Photichthyidae) (Wilson & McCormick 1997, 1999; McCormick *et al*., 2002). Settlement is a transitional event that is frequently evident in otoliths and of interest in ecological studies of juvenile fish (Green *et al*., 2009). Settlement marks, can be gradual or abrupt, are frequently species-specific, and can vary within species among local populations (Wilson & McCormick, 1997, 1999). Studies on recruitment of fish in the tropics often rely on the measurements of settlement marks. Growth rates of pelagic larvae which have been argued to be one of the principal determinants of recruitment success have also been estimated from otolith's microstructure (Bergenius *et al*., 2002).

Otoliths grow by successive deposition of increments, the so-called primary increments, which constitute bi-partite structures each composed of one L-zone and one D-zone. The L-zone is a band rich in calcium carbonate crystals, translucent to light (LM) and appearing raised in scanning electron microscopy (SEM), with an increment width varying between 0.4 and 10 μm. The D-zone is a band rich in organic material, opaque to LM and appearing as a groove in SEM, with an increment width smaller than 1 μm (Panfili *et al*., 2002). The width of a primary increment (also named micro-increment) usually ranges between 1 and 12 μm (Pannella, 1974).

Micro-incremental patterns in the otoliths vary from sub-daily to daily, lunar and seasonal scales (Campana & Neilson, 1985). Rhythmical patterns in the deposition of increments in the otoliths of fish are the basis of age estimation and depends of an endogenous rhythm externally calibrate by the photoperiod (Morales-Nin, 2000). The influence of lunar rhythms in the recruitment of coral reef species has been demonstrated since it alters the otolith microstructural pattern (Sponaugle & Pinkard, 2004). Food intake and food deprivation also have an influence on microstructure, width and periodicity of increments in otoliths (McCormick & Molony, 1992; Molony, 1996; Massou *et al*., 2002). Stress-induced marks indicate the cessation of otolith

growth, which is rare phenomenon, and appear under transmitted light microscopy as opaque, regular, thin marks (Pannella, 1980).

Salinity or dissolved oxygen concentration are exogenous factors potentially affecting micro-increment periodicity, but they have scarcely been studied. It is known that salinity induces changes in otolith composition at the microstructure level, yet little is known on how salinity can affect microstructure deposition (Green *et al*., 2009). Oxygen depletion in the water has been hypothesized to cause disruption of microstructure deposition in *Tilapia sp.* (Pannella, 1980) and even resorption of the otolith edge in *Carassius auratus* (Mugiya & Uchimura, 1989).

The micro-chemical composition analysis has added another dimension to otolith studies (Green *et al*., 2009). Geochemical signatures encoded within otoliths are natural tags that are continuously recording information throughout the lifetime of a fish and can be used to infer differences in a range of ecological characteristics of a species such as migration history (Radtke *et al*., 1996; Tzeng & Tsai, 1994), temperature history of fishes (Radtke, 1989; Radtke *et al*., 1990; Townsend *et al*., 1995) and differentiate among fish stocks (Campana *et al*. 1994; Milton *et al*., 1997; Thorrold *et al*., 1998b). Otolith chemistry has been used to validate microstructural features in otoliths such as annual increments (e.g. Kalish, 2001; Andrews *et al*., 2005), metamorphosis and settlement marks (e.g. Arai *et al*., 1997; Shen & Tzeng, 2002).

Inductively coupled plasma mass spectrometry (ICP-MS) was introduced in 1980 and was originally conceived for solution-based analysis but laser ablation devices were quickly developed that could be coupled with ICP-MS instruments to provide *in situ* analyses of otoliths. The ability to analyze multiple elements in otoliths at concentrations down to ultra-trace levels has led to new insights into larval dispersal pathways in both tropical and temperate oceans (e.g. Swearer *et al*., 1999; FitzGerald *et al*., 2004). New techniques have been developed that use the otolith as a storage device for unique transgenerational isotope labels rather than as a record of natural environmental variations (Thorrold *et al*., 2006).

1.3. Objectives

The main purpose of this work will use the microstructure of otoliths to improve the knowledge about some life history traits of *L. pholis*. Firstly, the primary increments deposition growth rate will be validated through the use of fluorescent markers (alizarin and tetracycline) in controlled-laboratory experiments; Secondly, the relationship between fish length and otolith size (radius and diameter) or total age will be explored; Thirdly, by counting the primary increments and by tracking the settlement mark on sagittae, the age of individuals at coastal recruitment will be estimated; and Fourthly, the relationship between the available record of the coastal Portuguese sea-water temperatures and the age at coastal recruitment in *L. pholis* will be assessed.

2. Materials and methods

2.1. Validation of otolith daily increments in early juveniles of shanny *Lipophrys pholis*

Fish Sampling and Acclimation

The fish sampling took place in Cabo do Mundo (41º13'N, 8º42'W) in March and August 2013. A total of 90 early juveniles were collected using a small hand net in the pool-tides during the low tide periods. The individuals were transported in refrigerated box with continuous oxygenation to the laboratory and kept in quarantine for two weeks to use in two independent experiments.

Experimental Design

Forty five recruits (TL of 19.9 ± 2.3 mm; range 17-23 mm) were placed in a 25 liters aerated seawater aquarium, followed by a buffered bath containing alizarin red S (Sigma-Aldrich, A5533, 100 mg/L) and another 45 individuals (TL of 26.1 ± 5.3 mm; range 16-39 mm) in tetracycline hydrochloride (Sigma-Aldrich, T3383, 400 mg/L) for 24 hours. The aquarium was kept in the dark to prevent light-degradation of the fluorescent chemical and the fishes were unfed and undisturbed. Bath immersions were buffered by adding potassium hydroxide (KOH, ≥85%) solution to adjust pH to

8.41 and 8.02, respectively. After 24 hours, the fish were removed and placed in three different seawater aquaria (replicates) with about 25 L of seawater, fed daily "*ad libitum*" with frozen shrimp and fresh mussels and with a controlled photoperiod of 12D:12N. During the experiments all tanks were monitored daily for water temperature, pH and salinity (Table 1), using a multi parameter probe (YSI, 556 MPS).

Fish were sacrificed following 10, 20 and 30 days of exposure. Otoliths from individuals that died before the end of the experiments were not used.

Otolith Preparation and Observation

Both sagittal otoliths were carefully extracted with plastic forceps under a magnification of 15x binocular stereo microscope (Meiji, EMZ-13TR) and cleaned with 70% ethanol to remove all organic tissues. Left otoliths were mounted on microscope glass slides with the convex side up using a drop of epoxy resin (Buehler, EpoThin). Resin was allowed to dry at room temperature during 24 hours. Otoliths were grounded with silicon carbide paper (Hermes, 2500), wetted with Milli-Q-Water and moving circularly the glass slides. During this procedure frequent checks were made in light microscope (Olympus, CX41), using a clearing mixture (ethanol:glycerol, 1:1), to see if micro-increments were most visible until the core was revealed. At the end, otoliths were polished with alumina paste (Struers, AP-Paste).

The detection of the fluorescent band was carried out by viewing the otolith through a compound microscope (Leica DM6000B) with UV light resource at 200x and 1000x, and thereafter viewed again under light microscopy to enumerate the number of growth increments between the fluorescent mark and the otolith edge (Fig. 6). Microphotographs were taken using a USB digital camera (Olympus, SC 30) and otolith morphometric measurements were made using a free software program (Olympus, MeasureIT SC30).The UV filters used were Leica filter N2.1 for alizarin red and Leica filter D for tetracycline. The number of increments was blind counted by three independent readers. The coefficient of variation was lower than 10%. The otolith growth rate has also estimated by measuring the maximum radius between the fluorescent mark and the otolith edge and dividing by the time elapsed.

Table 1- Abiotic parameters controlled in seawater aquaria during the otolith validation studies (mean ± standard error).

		Abiotic Parameters		
	Replicates	Temperature (ºC)	Salinity (psu)	pH
Alizarin	1	16.2±0.5	35.3±0.6	7.96±0.06
	2	16.0±0.5	35.3±0.6	7.92±0.05
	3	15.8±0.5	35.4±0.6	7.98±0.06
Tetracycline	1	16.1±0.2	35.0±0.6	8.74±0.06
	2	15.7±0.2	35.0±0.6	8.75±0.05
	3	15.1±0.2	35.0±0.6	8.77±0.04

2.2. Age at costal recruitment inferred from the otolith microstructure of early juveniles of shanny *Lipophrys pholis*

Biological Sampling

Three sampling campaigns were conducted in April/ May (2013) and a total of 91 young juveniles fishes were collected in three rocky beaches equally spaced along the Portuguese coast from North to South (Cabo do Mundo: 41º13'N, 8º42'W, Peniche :39º26'N, 9º13'W, and Vale do Homem : 37º22'N, 8º49'W) (Fig. 3). Since temperature seems to influence the growth of the species, the sea surface water temperatures in these regions were retrospectively considered for 3 months (average mean age of the individual captured). These data were available from the Instituto Hidrográfico da Marinha Portuguesa (Table 2). The specimens smaller than 30 mm (recruits) were collected with hand-nets in rocky pools during the low-tide period. The recruits were kept in sea-water, placed in ice and rapidly transported to the laboratory. All fishes were measured (total length: TL, mm) and distributed by size classes at

Figure 3 - Portuguese Rocky beaches where recruits were collected (modified from Google Earth)

intervals of 1mm (Fig. 4). The individuals from each size class were frozen (-20ºC) together in Eppendorf tubes filled with seawater.

Table 2 – Surface sea water average temperatures (ºC) in three points of Portuguese coast in March, April and May 2013 provided by Instituto Hidrográfico da Marinha Portuguesa (nm, means nautical miles).

		Sea Surface Temperatures (°C)			
Buoys position	Coastline distance (nm)	March	April	May	Mean
Leixões	11	12.7	13.6	13.0	13.1
Nazaré	4	13.2	14.0	14.0	13.7
Sines	3	14.5	15.5	15.5	15.2

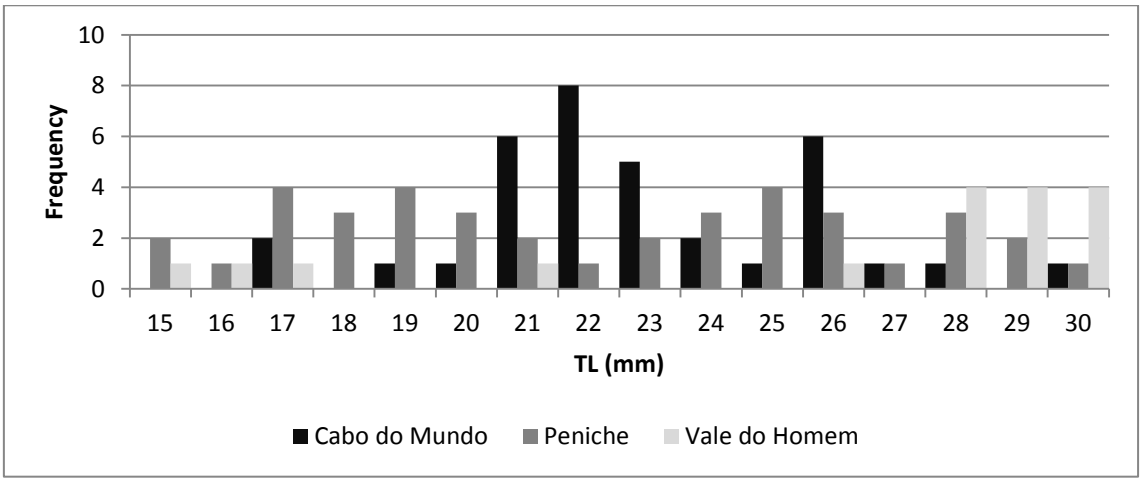

Figure 4 - Frequency of fish sizes captured in Cabo do Mundo, Peniche and Vale do Homem (n=91).

Otolith Preparation and Observation

Otoliths were carefully extracted and cleaned with Mili-Q-Water. Left otoliths were mounted on microscope glass slides with the convex side up using a drop of epoxy resin (Buehler, EpoThin). Whole otoliths were photographed in a light microscope (Olympus, CX41) coupled to an USB digital camera (Olympus, SC30) at 200x and 400x magnification. The radius, diameter and increment width of sagittae were measured (µm) in their longest axis using a free software program (Olympus, MeasureIT SC30).

After the measurements, the otoliths were manually grounded in the sagittal plane with silicon carbide paper (Hermes, 2500) and polished with alumina paste (Struers, AP Paste).

Final images were worked using a computer program (Olympus, AnalySIS getIT SC30). If needed, successive series of microphotographs from each otolith were made to obtain a complete image of the otolith radius.

To count the micro-increments the quality of the otolith's images were sharpen using a free software (GIMP2.8.4).To determine the total age of recruits at the moment of capture, 16 days were addicted to the counted primary increments (Faria et al., 2002), which corresponds to the number of days before hatching (embryonic period). It was assumed that the first daily ring should represent the hatching check. The number of daily increments was blind counted by three independents readers. Only otoliths in which variation coefficient were lower than 10% were used (average counts).

Settlement marks in the otolith were visually identified by the same reader using the optical density transitions and the abrupt change in increment widths (McCormick, 1994).

2.3. Statistical analysis

Statistical analyses were performed using SigmaPlot 11. All analyses were carried out according to the common statistical procedures (Zar, 1996). Analysis of covariance (ANCOVA) was used to compare replicates (linear regressions slopes), in alizarin and tetracycline experiments. Since there was no significant differences in replicates ($P>0.05$), only one linear regression for each treatment was used for data analysis. To assess the daily growth increment periodicity, the slopes of the linear regressions were tested through one sample t-test ($H_0=1$).

Linear regression between otolith measurements (radius and diameter) and total length of recruits captured at Cabo do Mundo, Peniche and Vale do Homem were analyzed. The total length of recruits among locations was tested using one-way analysis of variance (ANOVA), followed by a Tukey post hoc test. Regression analysis was conducted on the recruits length and age at capture. ANOVA was used to explore the individual age at recruitment between locations, also followed by a Tukey post hoc test. A level of significance (α) of 0.05 was used. Data are presented as mean values ± standard deviation (S.D.).

3. Results

3.1 Validation of otolith daily increments in early juveniles of shanny *Lipophrys pholis*

Alizarin and tetracycline appeared as distinct bright red and yellow rings, respectively, when viewed under ultraviolet light in the otoliths of all early juveniles of *L. pholis* [Figs. 5a and 6a). Both marks were also clearly visible when viewed with normal light microscopy (Figs. 5b and 6b).

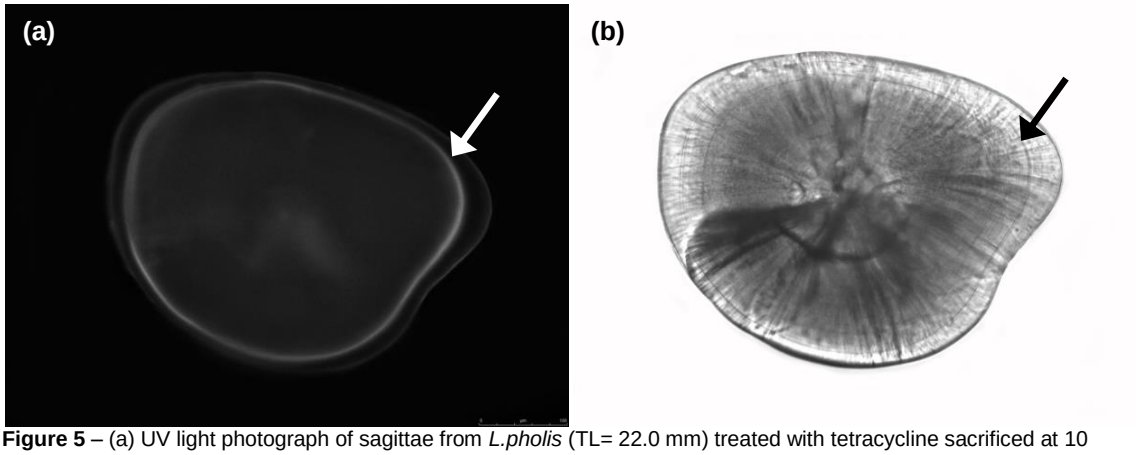

Figure 5 – (a) UV light photograph of sagittae from *L.pholis* (TL= 22.0 mm) treated with tetracycline sacrificed at 10 days. (b) Photograph of the same sample under normal light with tetracycline mark (200x)

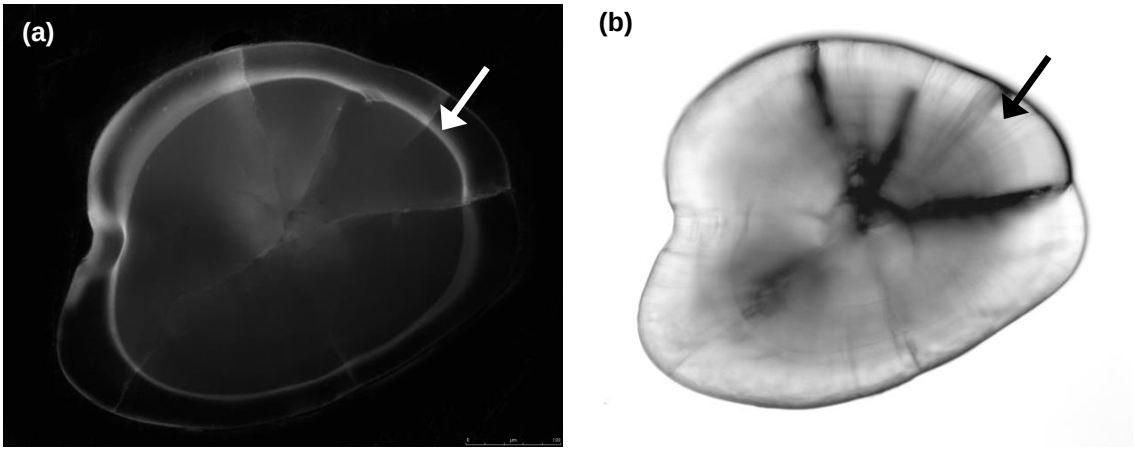

Figure 6 - (a) UV light photograph of sagittae from *L.pholis* (TL= 23.0 mm) treated with alizarin sacrificed at 30 days. (b) Photograph of the same sample under normal light with alizarin mark (200x)

No significant differences were detected among regression slopes (i.e. replicates) within each experiment (ANCOVAs: d.f. = 2.21, P > 0.05 and d.f. = 2.32, P > 0.05, for alizarin and tetracycline, respectively). The regression of number of

increments on time in the experiment for the individuals marked with alizarin was: y=0.96x - 0.3 (r^2= 0.98), and for individuals marked with tetracycline was: y=0.97x - 1.12 (r^2=0.96) (Fig. 7). The slope of the relationship was not significantly different than 1 (0.96 and 0.97, respectively for alizarin and tetracycline) (one sample t-test, d.f. = 5, P > 0.05).

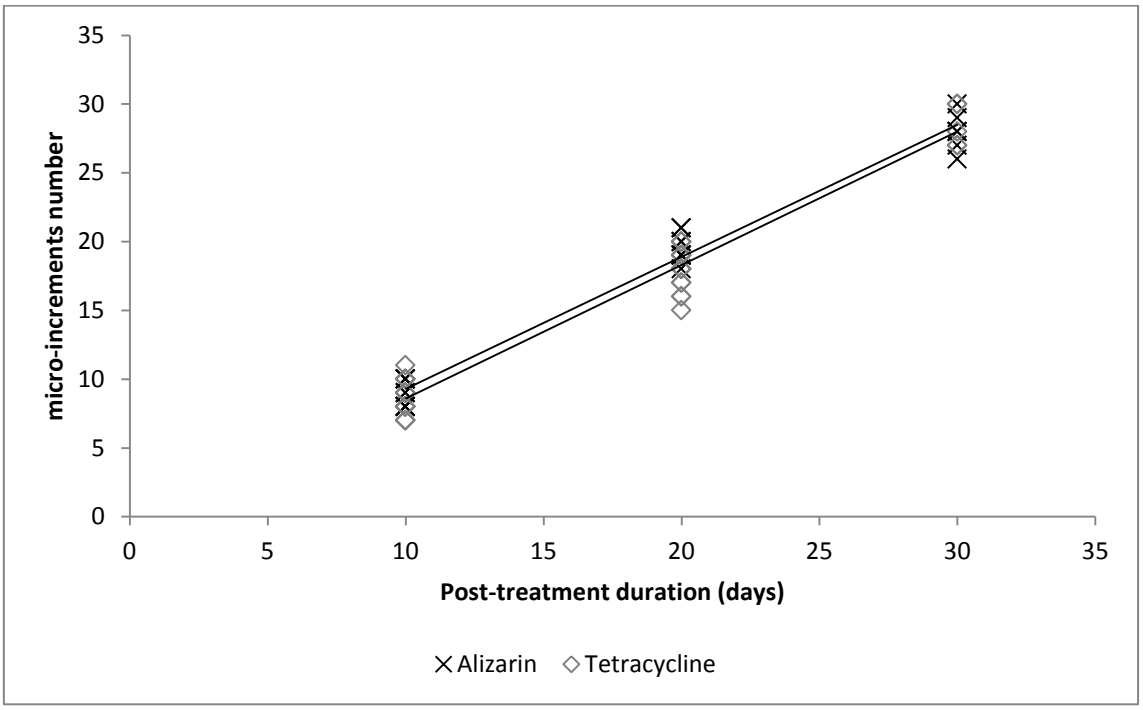

Figure 7 - Relationship between number of increments reading of marked otoliths, with alizarin (n=27) and tetracycline (n=38), and the number of days in the experiment.

The daily growth rate during the experimental period for *L. pholis* otoliths was 1.2 ± 0.3 µm/day for alizarin and 1.3 ± 0.3 µm/day for tetracycline, but there was no significant differences between the number of growth increments for both experiments (ANOVA, P<0.05). The overall growth rate was 1. 25 ± 0.3 µm/day.

3.2. Age at costal recruitment inferred from the otolith microstructure of early juveniles of shanny *Lipophrys pholis*

Significant differences were observed in the mean lengths among L. pholis samples (One-way ANOVA: n=91, d.f. = 2.88, P < 0.05) (Table 3).

Table 3 - Information relative to the total number of captured individuals (n), Total Length in mm (TL: mean ± S.D.) and range (mm).

Site	n	TL (mm)	Range (mm)
Cabo do Mundo	35	23.0±2.9	17.0-30.0
Peniche	39	22.1±4.4	17.0-30.0
Vale do Homem	17	26.1±5.3	15.0-30.0

A positive significant correlation (Fig. 8) was found between the otolith measurements (radius and diameter) and the fish length (Radius: r^2=0.82, P < 0.05, n=91; Diameter: r^2=0.86, P < 0.05, n=91). The overall sagittae radius and diameters ranged from 138 to 323 µm and 263 to 588 µm, respectively. The relationship between the two variables is given by the equations: y = 9.96x + 11.26 and y = 18.66x + 25.65, respectively.

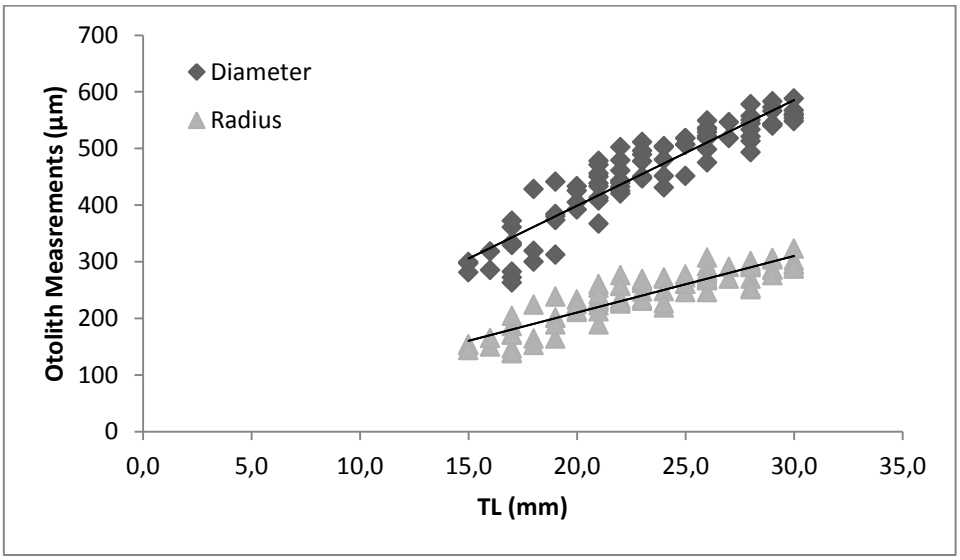

Figure 8 – Relationship between the radius and diameter with the fish length (p<0.05).

The estimated total number of increments (age at capture) from *L. pholis* captured at Cabo do Mundo, Peniche and Vale do Homem ranged from 73 to 125 days (21.1 ±1.7 mm), 66 to 99 days (18.4 ±1.9 mm) and 61 to 116 days (26.1 ±5.3 mm),

respectively (Fig. 9). The regression equation for relationship between the length of recruits and age at capture to Cabo do Mundo, Peniche and Vale do Homem were, respectively, $y=4.2002x + 13.186$ ($r^2=0.42$), $y=4.2472x + 2.1142$ ($r^2=0.67$) and $y=2.9533x + 16.924$ ($r^2=0.83$).

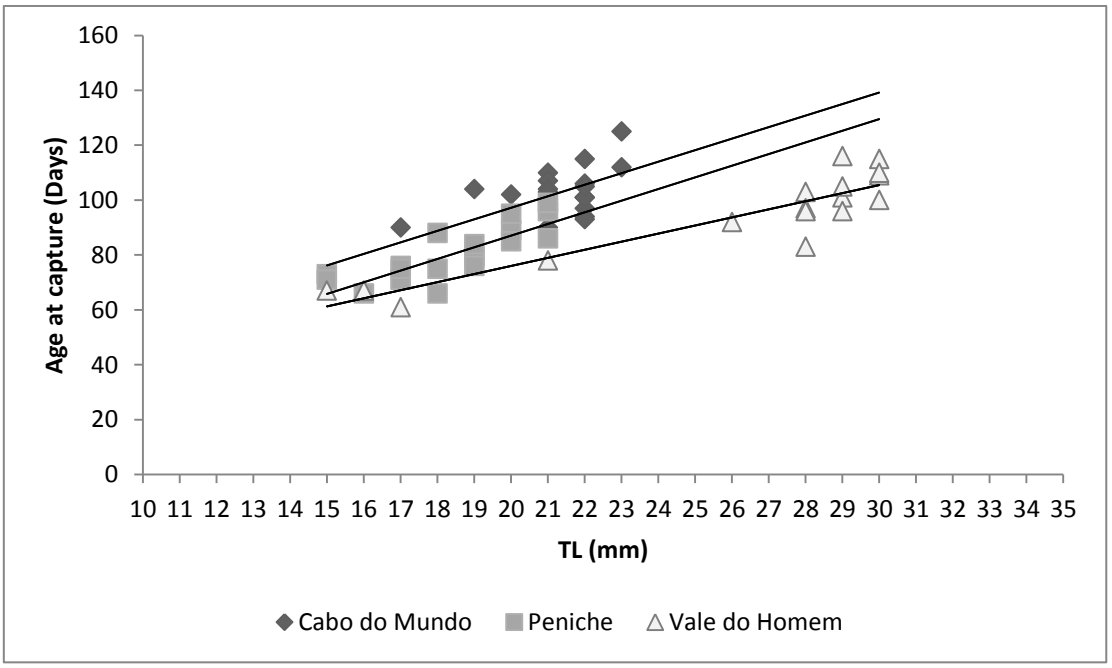

Figure 9 – Linear regression relating individual Length (TL) with age at capture from Cabo do Mundo (n=20), Peniche (n=20) and Vale do Homem (n=17)

Two settlement mark types were identified (type Ia and Ib) in all otoliths of captured recruits for the three sampling sites (Fig. 10).

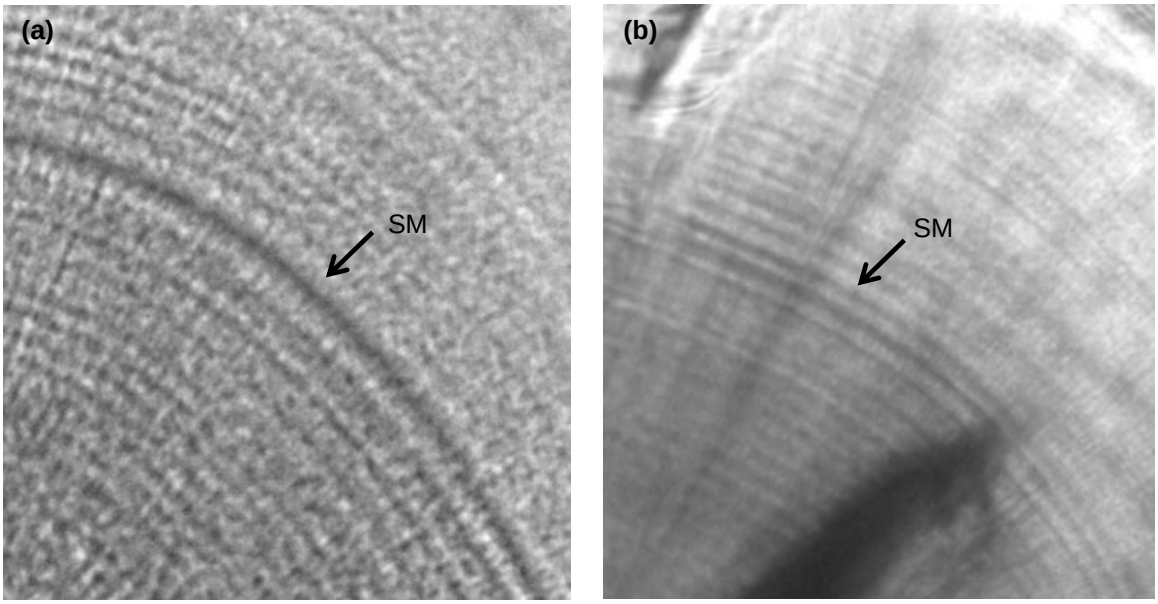

Figure 10 - Sagital sections of *L. pholis* otoliths: (a) Type Ia settlement mark; (b) Type Ib settlement mark.

There were significant differences in age at recruitment of *L. pholis* in Cabo do Mundo (73 ± 7 days), Peniche (62 ± 6 days) and Vale do Homem (57 ± 6 days) (One-way ANOVA: n=57, d.f. = 2.54, p<0.05).

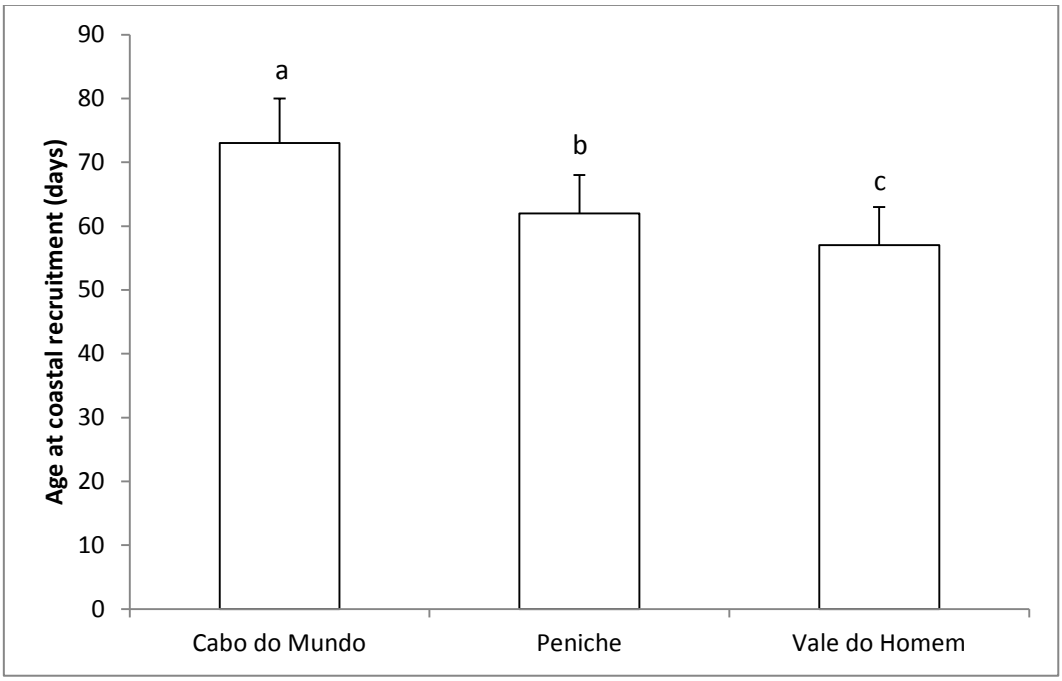

Figure 11 - Age at coastal recruitment (mean ± SD) in otoliths of *L. pholis* collected in three sampling locations (Cabo do Mundo n=20, Peniche n=20 and Vale do Homem n=17) along the Portuguese coast. The locations marked with different letters above the error bars are significantly different (Tukey-test, P<0.05)

There is a regular increase of increments width (1.08 - 3.25 µm) up to a distance of about 40 and 70 increments. Afterwards there is a decrease of width (1.45 - 3.31 µm) until the otolith edge up to a distance of 130 increments. The analysis of increment width confirms an increase in the width of the increments in the zone were the settlement occur which corresponds to 46 and 86 days before hatching, along the Portuguese coast (Fig. 12).

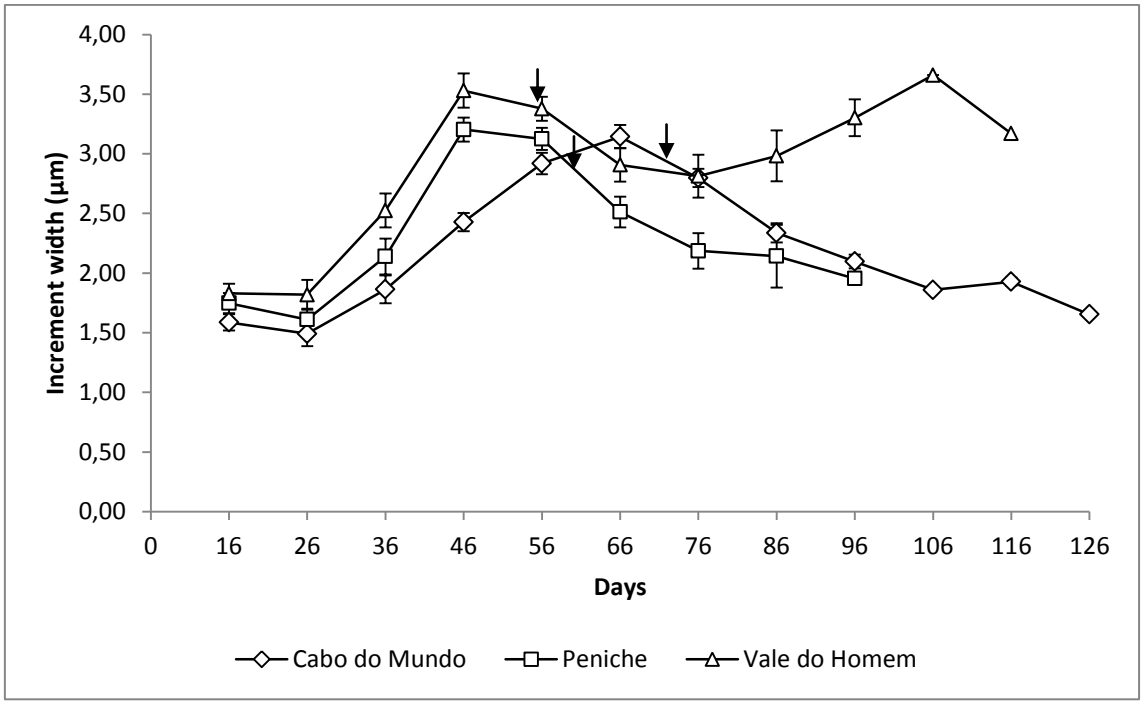

Figure 12 – Mean Increment width (µm) from primordium to the edge of the otolith increment. The individuals captured were grouped according to the sampling sites and SM was identified (↓). (Cabo do Mundo n=20, Peniche n=20 and Vale do Homem n=17) (Square error).

The mean daily otolith growth for *L. pholis* otoliths for the three sampling sites was 2.18±0.55 µm/day (Cabo do Mundo), 2.29±0.56 µm/day (Peniche) and 2.90±0.62 µm/day (Vale do Homem).

4. Discussion

Otoliths are chronological valuable tools for obtaining important ecological data for fish. However, it usually requires the previous validation of the deposition growth rate of the increments in otoliths (Beamish & Mcfarlane, 1983). This can be done in a direct manner involving marking of fish, through fluorochrome dyes or capture–recapture experiments, or by the indirect use of statistical techniques (Panfili *et al*., 2002). Chemical tagging is accepted as one of the best available methods to validate the periodicity of otolith increment deposition (Campana, 2001). Chemical tagging can be done through intraperitoneal injection, dietary intake and bath immersion, depending on the fish life stage and the environment (marine or fresh water) (Lagardére & Anras, 2000).

Marking of fish by immersion involves a compromise between chemical concentration, immersion period, water salinity, mortality rate, growing condition and retention time to produce the best mark (Taylor *et al*., 2005; Liu *et al*., 2009). Additionally it requires substantially less time and expense than previously reported techniques and seems to be the better option to young juvenile fishes, since the stress caused by handling is significantly reduced (Geffen, 1992).

Alizarin and tetracycline are the most used chemical markers (e.g. Lang & Buxton, 1993; McCormick, 1994; Vigliola, 1997). Although tetracycline seems to be the most common used chemical (Hernaman *et al*., 2000), it is also the most problematic treatment because of its toxic effects (Geffen, 1992). Mortalities rates are sometimes higher and the degree of incorporation in otoliths lower than calcein or alizarin (Vigliola, 1997).

In the present study, both dyes used in standard concentrations and immersion bath times, gave good results in producing a fluorescent visible mark in the otoliths of all juveniles. However, it was necessary to use a higher concentration of tetracycline to mark the same number of individuals. The fact that this chemical chelates with the divalent cations dissolved in seawater could be the reason (Hettler, 1984; Vigliola, 1997).

In this study, mortality resulted greater than reported in other similar studies, namely for alizarin red s [e.g. Beckman & Schulz (1996): *Cutovomin tommerfoni, Campoftoina anomalum* and *Plzoxinus erythrogaster;* alizarin red s, 100-200 mg/L, 3%-14%; Ibáñez *et al*., (2013): *Oreochromis niloticus*, alizarin red s, 50-100 mg/L, 12%; Unfer & Pinter (2013): *Salmo trutta*, alizarin red s, 150 mg/L, 13-31%]. The reported variation of the mortality rates between studies is most commonly attributed to species,

size of individuals and life stage of fish, more than to the high concentration of dyes and the duration of the immersion period (Hettler, 1984; Vigliola, 1997; Liu *et al*., 2009). Size of fish could be an explanation of the high mortality of alizarin in this study. Fish used for alizarin (19.9±2.3 mm) were smaller than that used for TC (26.1±5.3 mm) and are probably more vulnerable to the marking and handling procedure. However mortality rates of 35% in successful treatments were considered tolerable for hatchery mass-marking programmes (Eckmann, 2003).

Our study also showed a significantly very good relationship between the number of micro-increments in otoliths and the true age of individuals during the experimental marking period. Furthermore, the obtained slope of the linear regressions was very close to 1. These data clearly indicate that the primary increments in sagittae were deposited in a daily basis in *L. pholis*.

No reported validation studies exists on otoliths growth increment periodicity for species belonging to Blennidae. However, Related families, like small cryptobenthic fishes, belongs to gobbiidae family have been documented to present a daily increment formation in otoliths (Hernaman *et al*., 2000). Our data provides, for the first time, evidence that daily growth increments are reliable sources of age information for *L. pholis*. This information means that the otolith microstructure of *L. pholis* can be useful to understand the recruitment process and the connectivity and dispersal movements of the species.

Results from this study also indicate that under artificial conditions the daily growth rate of the otoliths for early juveniles of *L. pholis* was 1.25 ± 0.30 µm. Similar values was obtained for juveniles of and *Diplodus putazzo*, with a mean growth rate of 1.9 ± 0.5 µm/day and the same value for *Diplodus vulgaris* with 1.9 ± 0.5 µm/day (Villanueva & Moli, 1997). For *Odontesthes bonariensis*, the mean growth rate was 3.3 ± 1.2 µm/day (Brown & Fuentes, 2005) and for juveniles of *Limnothrissa miodon*, ranged from 1.6 to 3.4 µm/day (Meisfjord, 2006).

Microstructure analysis of *L. pholis* otoliths represents an efficient tool for the analysis of life history traits, and provides a range of information that had not yet been obtained for this specie.

The age, size and body composition of a newly metamorphosed fish represents the product of all planktonic influences experienced, such as temperature, feeding history and parentage (Green *et al*., 2009). The easiest way of overcoming differences in pelagic life histories experienced by larvae when attempting species comparisons is by collecting samples over a wide range of environmental conditions. This will be

particularly important for fishes with long larval durations and high associated variability (Victor, 1986).

The otolith measurements (radius and diameter) in this study showed a positive relationship between fish length. As expected, and described by other authors, fish size and otolith length are related (Alhossaini & Pitcher, 1988; Hovenkamp, 1990; Hovenkamp & Witte, 1991). Because of their function in maintaining the balance of the fish, otoliths tend to grow as the fish grows (Campana, 2004).

Many marine organisms, including cryptobenthic fishes, have complex life cycles divided in two main phases: i) the larval stage, which is generally spent in the plankton as gametes and larvae, ii) and the juvenile and adult phases, which starts when the larvae settles into benthic habitats (Roughgarden *et al*., 1988). Between these phases there is a transition period (i.e. settlement), which is characterized by more or less abrupt morphological and physiological changes (Leis, 1991).

Specific variation in the environment can induce a specific mark in the otolith structure which could be used as a reference mark to check some fish life history traits (Sponaugle & Pinkard, 2004). Settlement is one transitional event that is frequently evident in the otoliths and of interest in ecological studies of juvenile fish and can be recorded in very different ways (Green *et al*., 2009).

Otolith settlement marks in reef species can be grouped into categories based on changes in increment width and optical qualities of the mark (Wilson & McCormick, 1999). Fishes with abrupt changes in otolith increment width associated with settlement (Type I) were found to settle during a period of a decrease otolith growth, a few days after reaching their peak growth. The peak in otolith growth may represent the time at which the otolith can function efficiently to meet a species' sensory requirements while in the pelagic realm (Wilson & McCormick, 1999).

In our study, two settlement mark types were identified, namely *Ia* and *Ib*, both characterized by a sharp decrease in increment width across the settlement mark completed within a few increments. These different types of settlement marks observed in *L. pholis* corresponds to a single transition increment (type *Ia*) or a few transition increments (type *Ib*) (Wilson & McCormick, 1999).

Beldade *et al*. (2007) investigate 10 temperate cryptobenthic species belonging to three families: Gobiidae, Gobiesocidae and Blenniidae. Two subtypes of settlement marks (type Ia and Ib) were found among individuals of the same species.

A similar scenario was also observed for some species (e.g. *Acanthurus olivaceus*, *Pomacentrus amboinensis*, *P. wardi*) which increment width decreases as much as 50% around the settlement-mark (Wilson & McCormick, 1999). In total, 80%

of the species examined for Wilson & McCormick (1999) displayed this dramatic reduction in increment width over settlement (characteristic of Type I).

Fundamental knowledge of dispersal and connectivity of aquatic organisms can be useful to understanding the biological and hydrodynamic processes involved in the transport of larvae and deriving larval origins. Natal origins and destination points provide the basic data in connectivity studies (Cowen, 2007). Time scales of larval development and behavioral capabilities, including vertical migration, play an important role in the evolution of life history strategy of individuals (Sponaugle *et al*., 2002; Cowen, 2002). Population connectivity of benthic marine organisms occurs primarily during the pelagic larval stage when individuals either return to their natal location to settle, or disperse and settle some distance away from their natal population (Cowen, 2007). Reconstructing settlement patterns and recruitment pulses can be used to estimate persistence of cohorts in populations and infer the relative importance of recruitment processes to adult's population sizes and dynamics (Victor, 1983).

The number of daily increments before the settlement mark indicates an age at recruitment of 73±7 days, 62±6 days and 57±6 days in north, center and south, respectively. Our results clearly reveal a latitudinal pattern of coastal recruitment, probably related with the seawater temperature. The total age for recruits ranged between 61 and 125 days.

McCormick (1994) examined the variation in the size of *Upeneus tragula* at settlement over 8 samples from single station off Lizard Island during November and December 1991. Fishes that settled early in the season were found to be significantly smaller than fishes that settled later (November: 26.9 mm; December: 28.1 mm). Furthermore, the mean age at recruitment did not differ between months (32.2 and 31.4 days respectively).

The Gobiidae studied by Beldade *et al*. (2007) presented a mean pelagic larval duration (DPL) ranging from 19 to 30 days. The only Blenniidae captured in the study, *P. pilicornis*, had the longest DPL among the species analyzed (33 days).

The effect of temperature on the growth of the otolith has been intensively studied and generally shows a positive relationship between otolith growth and temperature (Campana & Neilson, 1985). The possible roles of photoperiod, air and sea temperatures in the control of reproduction in *L.pholis* were investigated (Shackley & King, 1977). These authors concluded that both photoperiod and temperature seem to affect the course of the annual cycle of gonadal maturation. More recently it has been suggested that the temperature is a more effective factor than photoperiod in triggering the onset of reproduction in this species (Almada *et al.,* 1990a).

The relationship between otolith growth and environmental factors was evaluated by comparing age at recruitment of *L. pholis* and the seawater temperature between sampling sites. As has been done for pomacanthids (Thresher & Brothers, 1985), labrids (Victor, 1986) and pomacentrids (Wellington & Victor, 1989), temperatures experienced by larvae should be reported, to enable comparisons of larval duration among geographic regions.

The sea surface water temperature seems to influence the growth of the species. In this study the water temperature was considered for coastline distances between 3 nm and 11 nm, but in the tide pools this temperature can have abrupt daily and or sazonal changes. The temperature in our study showed a variation (Vale do Homem > Peniche > Cabo do Mundo: 15.2 ° C > 13.7 ° C > 13.1 ° C) in the period between March and May 2013. In regions with higher seawater temperatures young juveniles recruit earlier to the coasts. For instance, one individual of *L.pholis* with a TL of 21.0 mm in the north, centre and south have different ages of recruitment. It means that in the northern fishes has a slow growth comparatively with the most southern individuals, meaning that they settled later. We can conclude that the temperature can influence the fish growth.

The temperature is considered an important factor in the early life stages of fishes and has influence in recruitment (Hovenkamp & Witte, 1991). For Hovenkamp & Witte (1991) settlement age is lower at higher temperatures, indicating that development appears to be more related with temperature conditions than with size of the individuals in settlement.

McCormick & Molony (1995) suggested that progeny spawned early the extended reproductive season and developing cooler water are likely to have slower growth rates, but may metamorphose and settle at an older age and larger size, than those spawned into warmer waters later in the season. This prediction is supported by latitudinal and regional differences in size or age at settlement suggested in other studies (Randall, 1961, Thorrold & Milicich, 1990).

Geffen *et al*. (2011) describes higher growth rates for juveniles of *Pleuronectes platessa* at higher temperatures, and according Alhossaini *et al.* (1989), the size reached in nurseries after the first growing period is also critical for recruitment.

Relative daily growth can be obtained by examining the width between successive increments. The comparison of increment widths during a particular stage of life among individuals provides a relative measure of somatic growth (Green *et al*., 2009). Increment widths measured in *L. pholis* otoliths showed variations between locations, there is an increase in width from north to south (Cabo do Mundo: 2.18±0.55

µm/day; Peniche: 2.29±0.56 µm/day; Vale do Homem: 2.90±0.62 µm/day). It means that fishes with more age (north) have thinner increments than younger fishes (south).

The patterns of daily growth of otoliths during early development, in relation to environmental factors, have been studied by time-series analysis of increment width data (Maillet & Checkley, 1991; May & Jenkins, 1992; Ralston, 1995).

Observations in the field and in the laboratory have shown that micro-increment width may change in response to temperature and diet, although the period of micro-increment deposition remained daily (Gauldie & Radtke, 1990). Food privation may result in smaller increment widths, but not in the cessation of microincrement deposition (Massou *et al*., 2002) since the fish has enough body energy reserves (Campana & Neilson, 1985).

Differences between increment widths before and after the SM were clear for *L. pholis*. Age at recruitment estimated by counting daily increments in otolith match with this differences in increment widths. Sagittae increment widths measured in this study are consistent with the increment widths reported for other fish families (Wilson & McCormick, 1999).

Studies on recruitment of fish in the tropics often rely on the measurements of settlement marks. Growth rates of pelagic larvae which have been argued to be one of the principal determinants of recruitment success have also been estimated from otoliths (Bergenius *et al*., 2002). The identification of settlement location is also possible using the differences in individual growth rates (Danilowicz, 1997a).

Future works using microstructural and microchemistry analysis from otoliths of individuals collected in different life stage (e.g. embryos, recruits and adults) can be used to assess the movement patterns and habitat connectivity in *L. pholis.*

5. Conclusion

This thesis have achieved to establish a daily deposition of the primary increments on sagittae of *L. pholis* suggesting that otoliths can be used to ageing purposes in this species. This information was used to estimate the age of *L. pholis* recruits collected along the Portuguese coast. Furthermore two types of settlement marks were identified by light microscopy. Larval stage duration appears to be highly dependent of seawater temperatures, but size appears to be a key process to trigger the settlement process. Reconstructing early life history patterns and recruitment pulses can be used to estimate persistence of cohorts in populations and infer the relative importance of recruitment processes to adult's population sizes and dynamics. Future studies should also use otolith microchemistry analysis to infer about the population structure and coastal connectivity of shanny.

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